Polyunsaturated Fatty Acids Help Regulate the Inflammatory Response in IPEC J-2 Cells Challenged with Enterotoxigenic *E. Coli*

Research with Distinction Thesis: Cameron White

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Abstract

The gastrointestinal (GI) tract plays a crucial role as a site for nutrient digestion and absorption in addition to serving as the largest immune organ in mammalian species. These functions are upended when the integrity of the intestinal epithelial barrier is disturbed. In the swine industry, the post-weaning period is characterized by stress-inducing situations, declining maternal antibody protection, and a shift from a liquid to solid feed diet; a trifecta that damages the gut barrier and leaves piglets susceptible to GI inflammation and infection. In fact, post-weaning enterotoxigenic *Escherichia coli* (ETEC) diarrhea contributes to high mortality rates on farms who must then incur all the costs associated with those losses. As antibiotic resistance remains a growing concern in animal agriculture, the discovery of alternative solutions becomes increasingly important. The objective of this research is to further evaluate the potential therapeutic role of dietary fatty acids in regulating intestinal inflammation and intestinal barrier function in pigs. The specific fatty acids examined are arachidonic acid (ARA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and oleate. Neonatal intestinal porcine epithelial cells (IPEC-J2 cells) were cultured in DMEM/F12 media supplemented with 5% FBS, ITS, EGF and antibiotics. Cells were plated at a density of 5×10^4 cells/well in a 6-well plate and grown to 100% confluence. After reaching confluence, the medias including the individual fatty acids were incubated with the cells for another 96 hours and subsequently challenged with ETEC. After being challenged for 6 hours, the cells were harvested for Western Blot analysis of claudin-1 and GAPDH protein abundance. The study found that ETEC challenge reduced claudin-1 protein in IPEC-J2 cells compared to unchallenged cells (0.48 vs 0.78 ± 0.25 ratio of claudin/GAPDH, respectively, $P = 0.01$). Within the challenged cells, DHA had a trend to protect from the loss of claudin-1 protein compared to BSA challenged cells (0.83 vs 0.39 ± 0.29

ration of claudin/GAPDH, respectively, $P = 0.08$). In conclusion, ETEC challenge in IPEC-J2 cells reduces tight junction protein expression and DHA has a potential protective effect against claudin-1 degradation in the challenged state.

Introduction

Intestinal disease is a leading cause of death in neonatal animals across species. In the swine industry specifically, scours is cited as the source of around 10% of all pre-weaning death loss (USDA, 2012). This deadly diarrhea most often results from ETEC infection (Nagy, 2005). This concern is not only contained to pre-weaning piglets either as *E. coli* infection in the post-weaning phase is associated with an 11.6% morbidity rate, meaning that 11.6% of all swine exposed to these bacteria develop an associated disease (USDA, 2012). The ability of *E. coli* to wreak such havoc on these animals is due in large part to its ability to activate intestinal inflammatory responses which alter the permeability of the gut and overall function of the intestinal barrier (Johnson et al., 2010). This intestinal barrier is a key element in optimizing the function of the intestine and maintaining animal health. Specifically, tight junction proteins like claudin-1, occludin, and zona-occluden are important components of this barrier that work to selectively transport materials from the lumen of the gut across the epithelium (La Shen et al., 2011). As relatively recent legislation has required the reduction of antibiotics in animal production due to an increased prevalence of antibiotic resistance, a greater focus is needed on alternative solutions that will also effectively combat disease in these animals.

Dietary strategies may be one such solution that have taken the forefront of research into alternative methods. In the case of our research, we were interested in examining the potential of polyunsaturated fatty acids (PUFA) in regulating the inflammatory response and protecting the function of the intestinal barrier in piglet intestinal cells that were challenged with *E. coli*. PUFA

have previously been shown to limit the induction of inflammation as well as reduce damage associated with this process. This is likely due to the ability of their metabolites to act as ligands for transcription factors like PPAR-γ (Korbecki et al., 2019). These transcription factors then help to resolve inflammation through multiple mechanisms. Understanding these processes, we hypothesized that including certain PUFA in the diet would enrich the intestinal phospholipid membrane, thus protecting the barrier function of IPEC-J2 cells when they were challenged with ETEC. The specific PUFA tested were arachidonic acid (ARA), eicosapentaeinoic acid (EPA), docosahexanoic acid (DHA), and the monounsaturated fatty acid, oleic acid.

Methods

Cell Culture and Fatty Acid Enrichment

To test this hypothesis, the IPEC-J2 cells, neonatal pig intestinal cells, were cultured in DMEM/F12 media supplemented with 5% FBS, ITS, EGF and antibiotics. Cells were plated on a trans-well plate at a cell density of 5×10^4 cells per well. Over the course of three days, these cells were grown to 100% confluence, meaning that they completely covered the surface area of the trans-well. Once cells reached this stage, they were supplemented with a fatty acid solution of 30 μM of either ARA, EPA, DHA, or oleate and incubated for 96 hours. This time was previously determined to be the optimal length of incubation to allow for maximal membrane incorporation of the fatty acids, after which time no additional incorporation would occur.

Fatty Acid Analysis

After incubation, the concentration of fatty acid in the membrane was measured using gas chromatography-mass spectrometry. Concentration of fatty acids in the phospholipid membrane of cells was determined by using a protocol similar to what was described by Hess et al. (2008). Cells were scraped from the 6-well plates and lipids were extracted from these scraped cells

using the Folch Method (Folch et al., 1957). Phospholipids were specifically separated from other classes of lipids using silica solid phase extraction columns from Sigma-Aldrich. The fatty acids were methylated and analyzed by a 5890 Hewlett Packard gas chromatograph equipped with 100-m capillary column and a flame ionization detector. The peaks on the chromatogram were identified by comparing retention times with authentic FAME (Sigma-Aldrich). Fatty acid concentrations were calculated using C17:0 as an internal standard and concentrations were expressed as grams / 100 grams fatty acid.

Enterotoxigenic *E. coli* Challenge

In a separate experiment, after cells reached confluence and were enriched with fatty acids, the cells were challenged with ETEC at a 2:1 multiplicity of infection (MOI) ratio as previously reported by Johnson et al. (2010), meaning that there were two ETEC bacteria for every IPEC-J2 cell, and incubated for six hours. The ETEC strain 3030-2: K88ac, LT, Stb was kindly provided to us by Dr. Philip Hardwidge of Kansas State University. An ETEC colony was scraped from the Luria Broth (LB) agar plate and grown in 50 mL of LB solution for 6 hours at 37 °C and 250 r/min. The fermentation broth was then centrifuged and washed three times with PBS at 1600 × g at 4 °C for 10 minutes each. Bacterial cells were resuspended in 15 mL PBS and bacterial populations were estimated by spectrophotometry at 600 nm optical density (OD_{600}). Approximate dilution factors were then calculated based on the previously determined OD_{600}-CFU standard curve and ETEC was added to the media at the 2:1 MOI ratio. Transepithelial electrical resistance readings (TEER) were recorded prior to and following the challenge. TEER was measured with the EVOM2 voltohmmeter from World Precision Instruments (Sarasota, FL). This reading, which measured the resistance in ohms of a low frequency current passed through the cells, was indicative of the integrity of the membrane. After these readings, cells were rinsed

three times with cold 1 × PBS and lysed in 1 × RIPA lysis buffer, which contained 1% Triton-X100, 10 mM Tris-HCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Sodium Deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM PMSF (Phenylmethanesulfonyl fluoride) and 1 × Halt Protease Inhibitor Single-Use Cocktail (Thermo). Cell lysate was passed through a pipette 20 times to form a homogeneous lysate and was then sonicated for 10 seconds three times. Following sonication, samples were rested on ice for 5 min and then centrifuged at 14,000 × g for 15 min at 4 °C to separate cell debris from protein. The resulting supernatant was transferred to a new tube and stored at -80 °C for further analysis.

Western Blot Analysis

Protein concentrations were determined using the Pierce® Microplate Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific). Approximately 6 μg of protein samples were resolved on 10 % SDS-PAGE gels and then transferred onto PVDF membranes (Bio-Rad) via wet transfer at 70 V for 30 minutes in a cold room. Membranes were blocked with 5% skim milk dissolved in TBST (dH_2O, 1 M Tris-HCL pH 7.6, 5 M NaCl, Tween 20) at room temperature for 1 hour and then were incubated at 4 °C overnight against the corresponding primary antibodies: Claudin (Invitrogen) at a dilution of 1:2000 and GAPDH Rabbit Polyclonal Antibody (Proteintech) at a dilution of 1:5000. TBST was used to wash the membranes three times at 10 minutes per wash. Membranes were then incubated with the secondary antibody, Goat Anti-Rabbit IgG (Thermo) at a dilution of 1:5000 for one hour at room temperature. Membranes were again subject to a 10-minute wash three times. Blots were developed using the SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) and quantified using the iBright 1500 Imaging System.

Statistical Analysis

Data were analyzed using SAS by ANOVA. Different letters on any tables or figures presented indicate statistical significance of $P < 0.05$.

Results

The first trial that was run without ETEC challenge was to determine how specific fatty acid supplementation of the IPEC-J2 cells affects subsequent fatty acid incorporation into the phospholipid membrane. In Table 1, incorporation of all three PUFA was increased following incubation of the cells with a fatty acid solution. ARA increased from a concentration of 0.96% in the membrane to 5.47%, showing a 5.7-fold increase in incorporation. EPA began at 0.03% concentration and increased 41-fold to 1.23%, and DHA went from 0.02% to 0.42%, demonstrating a 21-fold increase in incorporation. These results indicate clear differences between fatty acid profiles in the phospholipid membranes of cells supplemented with specific fatty acid solutions, meaning that future differences in membrane integrity and protein abundance post-ETEC challenge could likely be attributed to this changing variable.

In a separate experiment where cells were once again grown and supplemented with fatty acid solutions before being challenged with ETEC, TEER values were measured before and 6 hours after challenge. There were no statistically significant differences of TEER values between the different fatty acid treatments, however there was a clear difference between TEER values before and after challenge (Figure 1). This demonstrates the negative effects of ETEC on intestinal barrier function as these lower resistance values indicate a damaged barrier. This decreased resistance also allows for greater ion flux across the intestinal membrane. Movement of ions into the lumen is a major factor in the development of diarrheal disease alongside ETEC infection as active absorption of nutrients and water is inhibited and active secretion of water into the lumen and out of the body is increased.

Figure 2 demonstrates the ratio of the tight junction protein claudin-1 to GAPDH, which is a housekeeping protein used as a control in this experiment as its expression levels are relatively unchanged by different treatments. Since its detection is the same across the gel, taking this ratio normalizes the protein expression of claudin-1. In almost all treatments, there is a decrease in claudin-1 abundance associated with *E. coli* challenge with a 0.48 vs 0.78 ± 0.25 ratio of claudin/GAPDH in challenged versus unchallenged cells, respectively, $P = 0.01$. However, comparing the claudin-1 expression in DHA supplemented cells compared to cells from the BSA control experiment, there is trend of increased claudin-1 in the challenged DHA treated cells compared to the challenged BSA treated cells. The ratio of claudin-1 to GAPDH BSA treated compared to DHA treated cells, respectively, was 0.83 to 0.39 ± 0.29, $P = 0.08$. More claudin-1 was detected when cells were supplemented with DHA, indicating a potential protective effect of DHA against claudin-1 degradation.

Conclusions and Future Research

In conclusion, by measuring fatty acid concentration before and after incubation with different fatty acid solutions, it was determined that supplementing PUFA can increase the amount of fatty acid incorporated within the membrane as compared to control cells by 5.7-fold with ARA, 41-fold with EPA, and 21-fold with DHA. By incorporating PUFA into the piglet's diet, we would expect to see increased phospholipid enrichment with the PUFA. With higher PUFA concentrations in the phospholipid membrane of the intestinal barrier, damage associated with ETEC or other pathogens would result in the release of these PUFA and synthesis of prostaglandin E2, thromboxanes, resolvins, and protectins, all metabolites that may help regulate the inflammatory response, leading to a more effective onset and resolution of inflammation in the intestinal tract. Optimizing macro- and micro-nutrient components in the diet and having a

well-managed environment could help swine producers lessen the impact of pre- and post-weaning opportunistic pathogens on intestinal health.

In addition, *E. coli* challenge clearly decreases TEER values of the IPEC-J2 cells' intestinal membrane, indicating decreased barrier function and increased ion flux across the membrane. Challenge with ETEC also demonstrates decreased expression of claudin-1 in IPEC-J2 cells, yet DHA showed potential protective effects against claudin-1 degradation as compared to the challenged control cells. The results of this experiment will be useful in future research to determine how abundance of other tight junction proteins like occludin and zona-occluden are similarly or differently affected by PUFA supplementation. In addition, it will be important to observe how transcription factors like NF-κB and PPAR-γ, which regulate the inflammatory pathway, are affected by fatty acid supplementation as metabolites of fatty acids are known to serve as ligands for these protein complexes. This research, and the opportunity it presents for future research, is crucial in furthering the investigation into dietary strategies that may serve as alternatives to antibiotic use in the swine industry.

Tables and Figures

		30 µM Treatment for 96 hours						
		Control	OA	ARA	EPA	DHA	SEM	*P*-value
% Fatty Acid in the membrane	OA	31.65^{ab}	32.26^{a}	29.56^{c}	30.11^{bc}	32.48^{a}	0.89	*P* < 0.05
	ARA	0.96^{b}	0.70^{b}	5.47^{a}	1.02^{b}	0.98^{b}	0.39	*P* < 0.05
	EPA	0.03^{b}	0.18^{b}	0.16^{b}	1.23^{a}	0.23^{b}	0.13	*P* < 0.05
	DHA	0.02^{d}	0.23^{c}	0.26^{c}	0.87^{a}	0.42^{b}	0.11	*P* < 0.05

Table 1: Fatty acid enrichment of isolated neonatal pig IPEC-J2 cells' phospholipids cultured in 30 mM ARA, EPA, DHA or oleic acid. Cells were cultured for 96 h in the presence of the BSA-without complexed lipids. The control cells were cultured in the presence of BSA. Values are means ± SEM, n = 6 wells of cells. Labeled means without a common letter differ, $P < 0.05$.

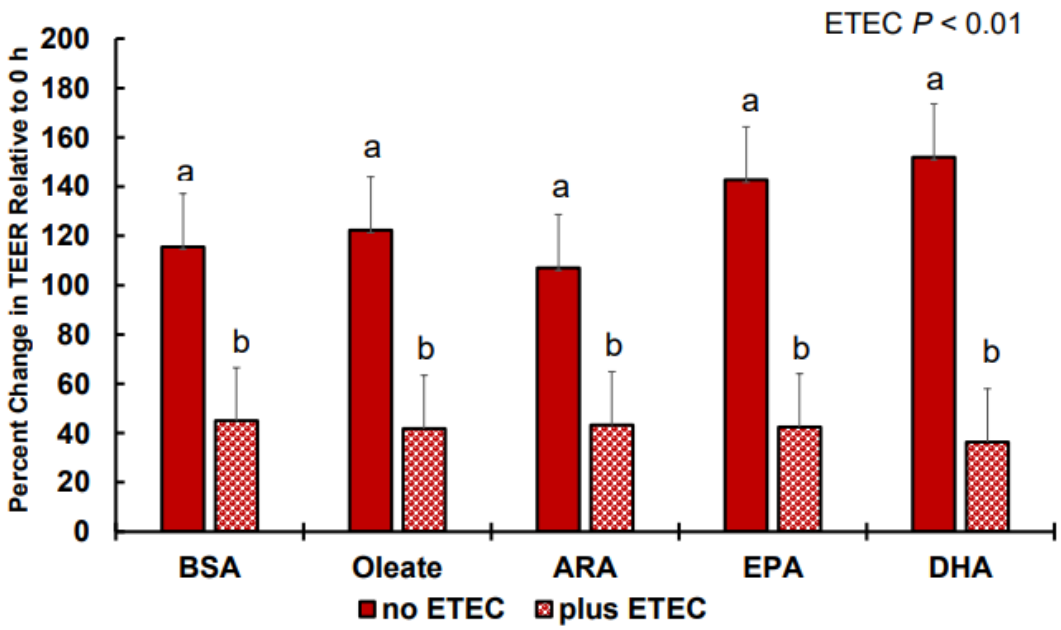

Figure 1: TEER measures of the IPEC-J2 cells monolayer previously enriched with 30 mM ARA, EPA, DHA, or oleic acid subject to a 6 h challenge with ETEC at a 2:1 MOI ratio compared to those without ETEC challenge. The control cells were cultured in the presence of BSA-without complexed lipids and then challenged with ETEC. Values are shown as a percent change relative to TEER values to the beginning of the challenge. Labeled bars without a common letter differ, $P < 0.01$.

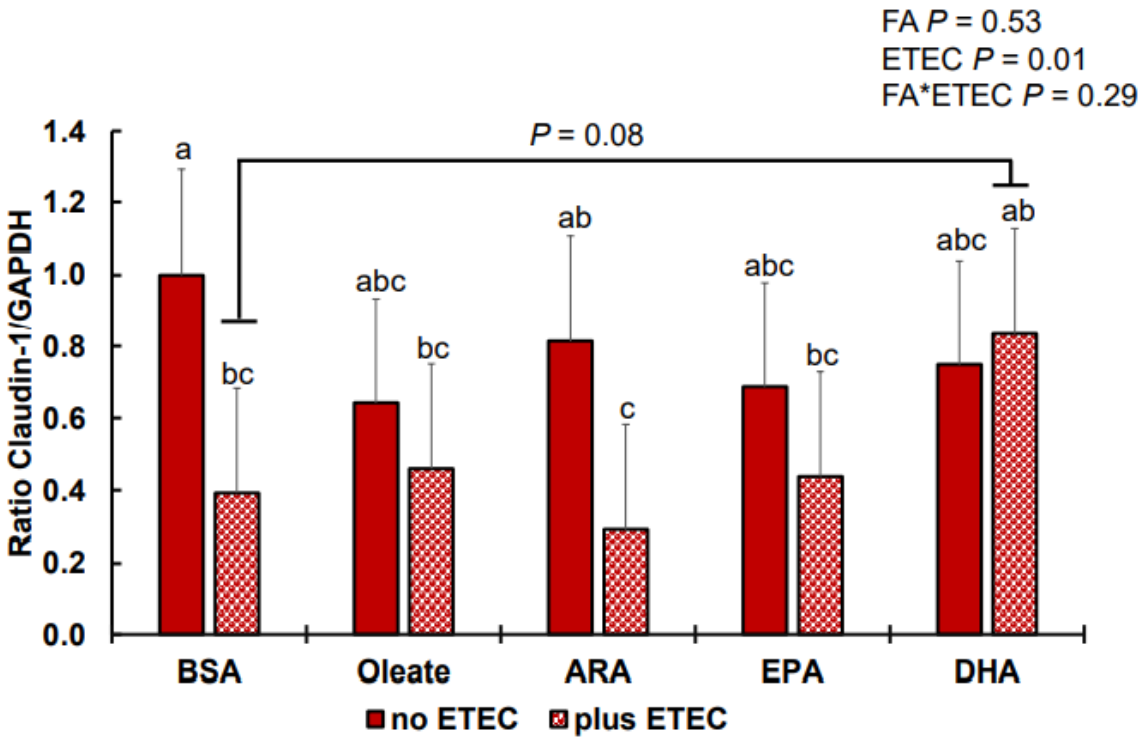

Figure 2: Ratio of Claudin-1 protein expression to GAPDH protein as measured by the iBright 1500 Imaging System in IPEC-J2 cells previously enriched with 30 mM ARA, EPA, DHA, or oleic acid subject to a 6 h challenge with ETEC at a 2:1 MOI ratio compared to cells with no ETEC challenge. The control cells were cultured in the presence of BSA-without complexed lipids and then challenged with ETEC. Labeled bars without a common letter differ, $P < 0.05$ unless otherwise specified in pairwise comparisons as labeled on the figure where reported ($P < 0.08$).

References

1. Johnson, AM, Kaushik,RS, Hardwidge PR. 2010. Disruption of transepithelial resistance by enterotoxigenic Escherichia coli. *Vet Microbiol*. 141(1-2):115-9. doi: 10.1016/j.vetmic.2009.08.020.
2. Folch J, Lees M, Stanley GHS. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*. 226:497–509.
3. Korbecki, J, Bobiński, R, and Dutka, M. 2019. Self-regulation of the inflammatory response by peroxisome proliferator-activated receptors. *Inflammation Research* 68:443–458. https://doi.org/10.1007/s00011-019-01231-1
4. Le Shen, CR Weber, DR Raleigh, D Yu, JR Turner. 2011. Tight junction pore and leak pathways: a dynamic duo. *Annual Rev Physiol*. 73:283-309. doi: 10.1146/annurev-physiol-012110-142150.
5. Nagy, B., and PZ Fekete. 2005. Enterotoxigenic Escherichia Coli in Veterinary Medicine. *International Journal of Medical Microbiology:* 295(6–7): 443-454. doi: 10.1016/j.ijmm.2005.07.003.
6. USDA, 2012. Part I: Baseline Reference of Swine Health and Environmental Management in the United States.